Exploration of the reproductive barrier between noug (*Guizotia abyssinica*) and its progenitor *Guizotia scabra ssp. schimperi*

Introduction

Domestication can be used to study the formation of barriers to reproduction because much is known about the selective forces at work during the process of domestication as well as the general timeline under which it occurs. Studying domestication may reveal how barriers to reproduction arise quickly and may give insight into speciation under natural conditions. Barriers to reproduction are very important in the context of domestication because this process is thought to occur in sympatry with the wild progenitor (Dempewolf 2011). To assess whether gene flow can readily occur between a crop and its wild progenitor, the entire reproductive barrier must be assessed. This barrier can be broken down into four parts. The pre-pollination barrier encompasses geography, habitat, mechanical and temporal isolation between the two species. The post-pollination pre-zygotic barrier includes the effects of pollen competition and genetic incompatibilities. If a hybrid is formed, it may be subject to intrinsic post-zygotic barriers of hybrid sterility and inviability. A surviving hybrid is lastly subject to external post-zygotic barriers which are reductions in fitness due to the external environment (Rieseberg & Willis 2007).

It is important to characterize the permeability of the reproductive barriers between crops and their wild progenitors for multiple reasons. Firstly, wild progenitors are excellent primary sources of germplasm for the improvement of crops and knowing the extent of the reproductive barriers between the two species can be invaluable to crop breeders. Secondly, as the use of transgenic crop lines becomes more prevalent in agriculture, knowledge of the reproductive barriers between the crop and its wild relative can be used to assess the risk of transgene escape. This is especially important when the transgenic crop is grown in its area of primary diversity, such as the growing of transgenic maize in Mexico (Gepts et al 2003), as the risk of transgene escape is much higher when many wild relatives to the crop are also growing in the area (Gepts et al 2003).

Noug (*Guizotia abyssinica*) is a semi-domesticated crop from Ethiopia. It is characterized as having a weak domestication syndrome as it is phenotypically similar to its progenitor, growing in a branching habit and producing small seeds from many heads. It is grown as an oilseed in Ethiopia, Eritrea and southern India and is exported to western markets as ‘niger seed’ for use as a birdseed (Getinet & Sharma 1996). Noug is sympatric with its progenitor *Guizotia scabra ssp. schimperii* (Hiremath & Murthy 1987), however a study using microsatellite markers suggests that the two species are reproductively isolated (Dempewolf 2011).

I propose to characterize the nature of the reproductive barriers that exist between noug and its progenitor. I will begin by conducting an observational survey of the flowering time in sympatric populations of domesticated noug and its wild progenitor. This experiment is particularly important, as pre-zygotic barriers are thought to make a greater contribution to reproductive isolation during species formation as they are often stronger and act before post-zygotic barriers (Ramsey et al., 2003; Lowry et al., 2008). Next, I will conduct two glasshouse experiments that explore post-pollination and post-zygotic barriers between the two species. I will use a pollination scheme to assess the competition between inter- and intra-specific pollen. I will then create artificial hybrids, measure traits relating to fitness and compare them to pure-bred half-sibs that I will also create. Finally, I will briefly describe field experiments that could further characterize the nature of the reproductive barriers that separate noug from its wild progenitor.

Experimental Design

a. Observational study of flowering time

Noug is very similar to its progenitor - an increase in seed size and oil quality within the crop are the main morphological differences between the two species (Dempewolf 2011). Ethiopian farmers do not recognize different strains of noug (Dempewolf 2011), but do recognize three general varieties based on maturation time (Petros et al 2007). Therefore, I predict that there may be differences in the flowering time between domesticated noug and its wild progenitor as there may have been artificial selection on this trait. To determine the extent to which the reproductive receptivity of the species overlap, I propose to observe sympatric populations of noug and its wild progenitor across the shared range in Ethiopia.

Noug is grown within regions of varying precipitation regimes and there is evidence that it may be locally adapted to these regimes (Dempewolf 2011), so it would be prudent to attempt to cover regions of varying precipitation, a environmental variable that is known to influence flowering time (Franks 2006). As the logistics of choosing specific study sites within a foreign country are complex, it would be difficult for me to specify particular study sites where I would observe sympatric populations. However I think it is important to attempt to cover the precipitation regimes found within the area in which noug is grown in Ethiopia. I will try and sample at least 10 population pairs for this experiment, although this number may vary depending on availability of populations and the logistics of monitoring the populations.

Noug fields that have a sympatric population of the progenitor species growing within ~500 meters could be used as study sites. A transect of specified length could be constructed through the densest portion of each population and plants (if possible) within 30 cm of the line could be observed over the course of the growing season. The measurements I propose to take on each individual are: date of first flower and the date when all flowers have matured.

I could use an ANOVA test that accounts for both population and species effects to determine if noug and its progenitor differ in their flowering schedules. I could also quantify the proportion of the flowering period that is overlapped between the two species and the proportion of the individuals of each species that are flowering concurrently with their congeners. I could then use this information to quantify the degree of reproductive isolation due to flowering time (RI_{flow}; Ramsey et al 2003). Finally, I could collect wild and domestic seed from my study sites and use them for my next two experiments.

b. Pollen Competition

Competition between pollen grains occurs when an excess of pollen contacts the stigma of the seed parent. Many studies, including one done using close relatives of noug - *Helianthus annuus* and *Helianthus petiolaris* have found that intraspecific pollen has a significant 'competitive edge' over interspecific pollen (Rieseberg et al 1995). This may function as an effective barrier to reproduction between the two species. I propose to conduct an experiment to investigate interspecific pollen competition as a reproductive barrier between noug and its progenitor.

I will follow the experimental design of Ramsey et al (2003) and create experimental crosses between noug and its progenitor using different ratios of intra- and interspecific pollen. As noug and its progenitor are closely related, I expect that the pollen of each species weighs approximately the same amount. However, I intend to use a hemocytometer to count pollen samples of the same weight from each species to ensure that the pollen is similar in size. Following the procedure, I will then make inter- and intra-specific crosses of varying proportions of pollen as shown in figure 1. Pollen from each species will be represented by five randomly chosen individuals (not within the maternal family of the seed parent) selected as pollen parents. I will prepare pollen mixtures by weight and apply them to the recipient flower using a paintbrush (following Rieseberg et al. 1995). As noug and its progenitor have complex flowers, I will repeat the pollinations three times to ensure that I have reached all the stigmas as they mature. I will also do a negative control where the flower is protected from pollination. As noug and its progenitor are self-incompatible, I expect that no seeds will be set in these treatments (Dempewolf 2011). I will choose three maternal families from each noug or progenitor population observed in experiment a. From each family I will grow one individual to be the seed parent for a total of 60 plants (30 noug and 30 progenitor).

noug	progenitor
0%	100%
25%	75%
50%	50%
75%	25%
100%	0%
0%	0%

Fig. 1 Varying proportions of pollen for use with seed parents of both species. The last treatment is a negative control where no pollen will be applied to the flower of the seed parent.

After the crosses are complete, I will count the seeds from each head and analyze the effects of the pollination treatment on seed set using an ANOVA test with a model that accounts for the effects of population and species (see Ramsey et al 2003). I will grow up the seeds from the mixed pollinations to determine the proportion of hybrids formed. As noug and its progenitor are very similar morphologically, I will have to determine hybrids based on previously characterized genetic markers (Dempewolf et al 2010) by genotyping the parents and offspring. If some of these markers are diagnostic of species, I will use them to genotype the progeny, otherwise I will have to use the most variable markers and a maximum likelihood approach to assess the paternity of the seeds (see Meagher 1986). I can then calculate the percentage of hybrids formed within each treatment and use an ANOVA test with an appropriate model to determine if there are differences in the proportion of hybrids among the pollen treatments for each species. I will then use this information to determine the reproductive isolation due to pollen competition for each species ($RI_{pollencomp}$; Ramsey et al 2003).

c. Glasshouse measurement of hybrid fitness & seed set

In my previous experiment, I crossed the noug and wild individuals with 100% inter- and intra-specific pollen. I will use the progeny from these crosses to assess hybrid fitness within a glasshouse setting. As the same seed parent was used to create both pure and hybrid progeny, I will be able to compare the hybrids with their pure-bred half-siblings.

I will grow up the seeds produced in these crosses and record the germination rate, survivorship to a chosen height and the percentage of plants within each cross that flower. I will collect a sample of pollen from each hybrid and corresponding pure-bred half-sib and assess pollen viability by staining with cotton blue (Kearns et al 1993 within Ramsey et al 2003). I will record the number of darkly stained pollen grains on a sample of 300 for each individual tested (Ramsey et al. 2003).

Assuming that enough of the F1 hybrids that I will create successfully flower, I intend to cross them in three ways and repeat my fitness measurements on another generation. I will use a similar method as above and do three different types of crosses on the chosen seed parents. Using different heads on the same F1 seed parent, I propose making backcrosses to each progenitor as well as a cross using pollen from F1 hybrids derived from the same maternal species. For each cross, I will collect pollen from five randomly chosen individuals of the correct pedigree and pollinate the chosen flower a total of three times.

From the data that I collect, I can determine the fitness of the F1 hybrid relative to the fitness of its pure-bred half-sib. By growing up an F2 hybrid generation, I can further track changes in fitness as possibly transgressive phenotypes may break down in later generations of hybrids. In addition, I will have the opportunity to examine backcrosses to

each parental species. This treatment is important because natural F1 hybrids may be more likely to mate with a member of either progenitor species than other hybrids. For each trait, I will use ANOVA with a model to account for maternal family effect to assess signifiant differences between the F1 and parental crosses. The traits of the F2 crosses can be examined in a similar way. I will then combine these measurements to get an estimate of lifetime fitness for F1 hybrids relative to parentals. I will then use this data to determine the degree of ($RI_{postzygotic}$; Ramsey et al 2003) for each species.

Discussion

I have considered logistics of studying non-local species throughout my proposal and have also considered the cost associated with traveling to another country. The study of flowering time within sympatric populations is the only part of my experiment that must take place in Ethiopia, and since it is only observational in nature and is an important component to the study. The other experiments that I have chosen can be accomplished in the glasshouse on campus which would make my proposal a plausible endeavor.

The experiments that I have described in my proposal can help to characterize the reproductive barrier that exists between noug and its progenitor. The observational study on flowering time in sympatric populations of noug and its progenitor assesses the pre-pollination portion of the reproductive barrier that exists between the two species. The reproductive receptivity of each species within the sympatric area must somewhat overlap for gene flow to occur in that area and this initial study tests whether this is possible. Domesticated noug has a long period of reproductive receptivity (Genet et al 2000), so I would not be surprised to find significant overlap in receptivity between the two species.

The pollen competition experiment tests the strength of post-pollination pre-zygotic barrier to reproduction between noug and its progenitor. A similar study was done using *H. annuus* and *H. petiolaris* and found that even when interspecific pollen outnumbered intraspecific pollen by a factor of nine, the majority of seeds set were sired by intraspecific pollen (Rieseberg et al 1995). This suggested that intraspecific pollen had a huge advantage when competing against interspecific pollen. As *Helianthus* is closely related to *Guizotia*, I would expect that intraspecific pollen would have a similar competitive edge. Both species used in my experiment are self-incompatible (Dempewolf 2011). This simplifies my experiment because I will not have to remove the anthers to prevent the self-fertilization within the seed parents.

As my described experiments explore both pre-zygotic and post-zygotic isolation, it is my hope that, used together, they may clarify what portion of the reproductive barrier is the strongest and this new knowledge can dictate future work on this topic. There are many more experiments that can be done to further our understanding of this reproductive barrier. First, reciprocal transplant experiments to assess immigrant inviability could be done (e.g. Wong et al 1995). Domestic and hybrid individuals could be transplanted into wild progenitor populations to assess survivorship within the ‘wild’

environment. The reverse transplant could also be done to assess the progenitor's ability to survive within a cultivated environment. This should be complemented by interviews with Ethiopian noug farmers to determine if they identify and remove noug hybrids or progenitors from their fields. This could be a very informative experiment, especially if the pollen competition study does not find evidence that intraspecific pollen outcompetes interspecific pollen.

Studying the nature of reproductive barriers in domesticated crops and their wild relatives can illuminate some aspects of natural speciation because we have an idea of the types and strengths of selection applied to the crop as it undergoes domestication. In addition, as more and more transgenic crop lines are being used, it is important to assess the relative risk of transgene escape.

References

Dempewolf, H. *Patterns of domestication in the Compositae and beyond.* Diss. University of British Columbia, Vancouver, 2011. Print.

Dempewolf, H., Kane, N. C., Ostevik, K. L., Geleta, M., Barker, M. S., Lai, Z., Stewart, M. L., Bekele, E., Engels, J. M. M., Cronk, Q. C. B, Rieseberg, L. H. 2010. Establishing genomic tools and resources for *Guizotia abyssinica* (L.f) Cass. - the development of a library of expressed sequence tags, microsatellite loci, and the sequencing of its chloroplast genome. Molecular Ecology Resources, 10(6): 1048-1058

Franks, S. J., Sim, S., Weis, A. E. 2006. Rapid evolution of flowering time by an annual plant in response to a climate fluctuation. PNAS 104(4): 1278-1282

Getinet, A. & Sharma, S. 1996. Niger, *Guizotia abyssinica* (L. f.) Cass. *Promoting the conservation and use of underutilized and neglected crops.* p. 59. International Plant Genetic Resources Institute Rome, Rome.

Gepts, P. & Papa, R. 2003. Possible effects of transgene flow from crop on the genetic diversity from landraces and wild relatives. Environ. Biosafety Res. 2: 89-103

Hiremath, S. C., Murthy, H. N. 1987. Domestication of niger (*Guizotia abyssinica*). Euphytica 37: 225 - 228

Kearns, C.A., Inougye, D. W. 1993. Techniques for pollination biologists. Univ. Press of Colorado, Niwot, CO.

Lowry, D.B., Modliszewski J. L., Wright, K. M., Wu, C. A., Willis, J. H. 2008. The strength and genetic basis of reproductive isolating barriers in flowering plants. Phil. Trans. R. Soc. B 363: 3009-3021

Meagher, T. R. 1986. Analysis of paternity within a natural population of *Chamaelirium luteum*. 1. Identification of most-likely male parents. The American Naturalist, 128(2): 199-215

Petros, Y., Merker, A., Zeleke, H. 2007. Analysis of Genetic Diversity of *Guizotia abyssinica* from Ethiopia using Inter Simple Sequence Repeat Markers. Hereditas, 144: 18 - 24

Rieseberg, L. H. and Willis, J. H. 2007. Plant Speciation. Science 317: 910-914

Wong, H., MacArthur E. D., Sanderson, S. C., Graham, J. H., Freeman, D. C. 1997. Narrow hybrid zone between two subspecies of big sagebrush (*Artemisia tridentata*: Asteraceae). IV. Reciprocal transplant experiments. Evolution 51(1): 95-102